Tissue granuloma structure-function in experimental visceral leishmaniasis

HENRY W. MURRAY
Department of Medicine, Weill College of Cornell University, New York, USA

Received for publication 22 May 2001
Accepted for publication 29 May 2001

Summary. In experimental visceral leishmaniasis in normal mice (BALB/c, C57BL/6) acquired resistance to *Leishmania donovani*, a protozoan which targets tissue macrophages, depends upon T cells, Th1 cell-type cytokine generation and activated mononuclear phagocytes. In the intact host, initial control and eventual resolution of *L. donovani* hepatic infection in normal mice is expressed by and accomplished within well-formed, mature tissue granulomas. In the liver, these immunologically active, inflammatory structures are assembled around a core of fused, parasitized resident macrophages (Kupffer cells) which come to be encircled by both cytokine-secreting T cells and influxing leishmanicidal blood monocytes. This pro-host defense granuloma structure-function relationship, in which histologically mature granulomas provide the microenvironment for intracellular *L. donovani* killing, however, is only one of seven which have been identified through experimental modifications in this model. This report reviews these structure-function relationships and illustrates the broad spectrum of additional possible responses. These responses range from structurally intact granulomas which provide no antileishmanial function (the 'ineffective' granuloma), to enlarged granulomas which show enhanced parasite killing (the 'hypertrophied' granuloma), to effective antileishmanial activity in the absence of any tissue reaction (the 'invisible' granuloma).

Keywords: cytokines, granuloma, monocytes, T cells, leishmaniasis, *Leishmania donovani*

Granulomas and visceral leishmaniasis: general considerations

Granulomatous inflammation, long recognized as a distinct histopathological reaction, can be induced by an array of stimuli including a variety of infectious diseases (Murray 1999). Capable of exerting largely beneficial but also potentially destructive actions, the granulomatous response apparently evolved in host antimicrobial defence as a specialized tissue mechanism quite separate from abscess formation. Thus, granulomas typically require an antigen-specific immune response mediated by mononuclear cells, develop relatively slowly and persist for extended periods, and

Correspondence: H. W. Murray, Box 136, 1300 York Avenue, New York, NY 10021, USA. Tel.: 212 746 6330; Fax: 212 746 6332; E-mail: hwmurray@med.cornell.edu

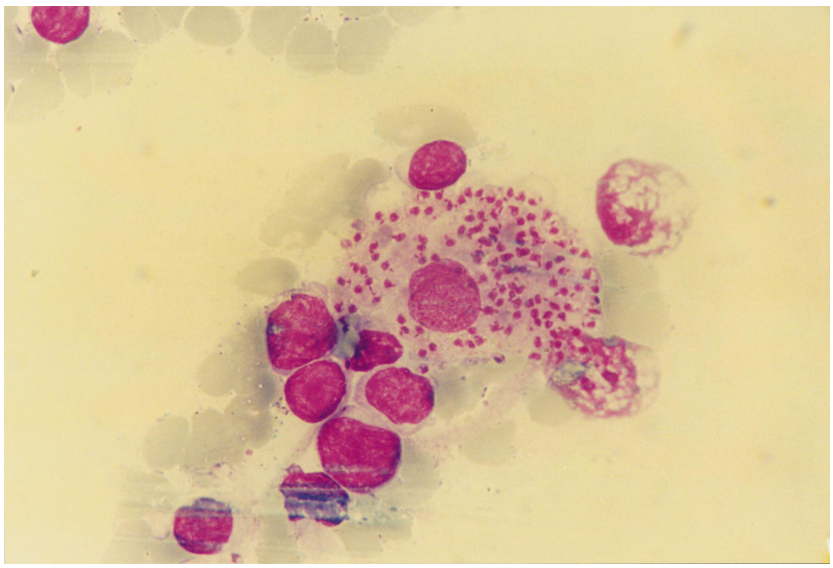

Figure 1. Giemsa-stained splenic aspirate smear from an Indian patient with visceral leishmaniasis (*L. donovani*) showing characteristic intracellular amastigotes within a splenic macrophage. (× 500). Aspirate slide was generously provided by Professor Shyam Sundar (Banaras Hindu University, Varanasi, India). Reproduced with permission (Murray, 2000a).

are more likely to be provoked by intra rather than extracellular pathogens.

Under optimal conditions, granulomas provide anatomically circumscribed, functional structures in which it is possible to limit infection, kill and remove the microbial target, and then repair any accompanying tissue injury. Intracellular infections associated with granulomatous inflammation include those caused by mycobacteria, select fungi and bacteria and certain protozoa such as *Leishmania donovani* (Adams 1976; Warren 1976; Williams & Williams 1983; Sheffield 1990; Murray 1999; Saunders & Cooper 2000). The latter pathogen preferentially targets resident visceral macrophages in the liver, bone marrow and spleen (Figure 1) (Murray 1999). In infections associated with granulomas, these structures are presumed to represent the tissue expression of the successful T cell-dependent immune response (Sheffield 1990; Mielke *et al*. 1997; Baker *et al*. 1999; Murray 1999; Hernandez-Pando *et al*. 2000; Saunders & Cooper 2000). In human visceral leishmaniasis, for example, the presence of granulomas in the liver appears to correlate with spontaneous control and maintenance of infection in a subclinical state (Murray 1999).

The overall antimicrobial efficacy of the granulomatous response appears to be variable, however, and depends upon host determinants, the pathogen and the criteria used to define efficacy. Although microbial eradication is the ideal outcome in infections which trigger granuloma assembly, it is not clear how often this objective is actually ever achieved. Thus, despite even an initially intense granulomatous response, a number of pathogens, including *L. donovani*, establish a well-recognized state of chronic intracellular (intragranuloma) parasitism and survive at low levels life-long. While microbial quiescense (walling-off) is attained under these conditions, such isolated tissue foci still require perpetual immunological reactivity and some form of granuloma maintenance or renewal to prevent recrudescence of latent infection. Relatively little is known about the underpinnings of this chronic granulomatous reaction which may persist for years.

In contrast, virtually all of the signalling mechanisms known to initiate cell-mediated inflammation, permit leucocyte trafficking from vasculature to infected parenchyma, and induce and maintain T cell-dependent responses can be detected in the events which lead up to granuloma assembly (Sheffield 1990; Anonymous 1991; Tapia *et al*. 1994; Adams & Lloyd 1997; Luster 1998; Baker *et al*. 1999; Murray 1999; Saunders & Cooper 2000; Marquez & Martinez 2001). New basic research insights and hypotheses related to initial mechanisms continue to be generated, attesting to the complexity of and unknowns in this response (Di Perri *et al*. 1999; Ehlers *et al*. 1999; D'Souza *et al*. 2000; Fallon 2000; Hesse *et al*. 2000; Saunders & Cooper 2000). Nonetheless, from an over-simplified but practical standpoint, the entire interlocking process of granuloma assembly can also be viewed as a straightforward mechanism which (a) delivers critical host defence factors, cellular and soluble, to the infected tissue site, and (b) then properly aligns, activates and retains these factors. Such an environment endows the host with selected effector cells (largely mononuclear) and a range of cytokines, growth factors and additional signals concentrated in one focus, yielding optimal induction and then maintenance of antimicrobial activity.

This same process can also go awry, however, if (a) the host is deficient in a required cell, activating cytokine or chemokine or adhesion signal, (b) an exogenous immunosuppressive treatment is given, (c) the pathogen itself stimulates a suppressive host response which deactivates primary granuloma-inducing mechanisms, or (d) if aberrant conditions lead to over-recruitment of inflammatory cells, including neutrophils (Murray 1999; Saunders & Cooper 2000). In addition, assembling a structurally intact-appearing granuloma does not necessarily guarantee antimicrobial function if the ultimate effector cell fails to become activated or is intrinsically deficient in a basic microbicidal mechanism. The effector cells within the granuloma in intracellular infections, including leishmaniaisis, are largely tissue macrophages and influxing blood monocytes; their activation, primarily by interferon-γ. (IFN-γ) and other inflammatory stimuli including tumour necrosis factor (TNF), is the final common pathway to the functional, antimicrobial state (Murray 1999; Murray 2001). The term, 'ineffective granuloma' (Murray & Nathan 1999), has therefore been applied to the setting in which macrophages within the granuloma remain heavily parasitized despite satisfactory kinetics of assembly and apparently intact cellular composition and structure.

Finally, at the opposite end of the spectrum of events which can derail the overall efficacy of granulomatous inflammation is failure of downregulatory mechanisms responsible for limiting tissue injury and terminating this tissue reaction. While certainly required to prevent uncontrolled, destructive and potentially fatal tissue inflammation, comparatively little is known about the signals which lead to granuloma involution in the infected host. Removing the primary stimulus by killing and digestion is a logical prerequisite, but subsequent molecular events, including pathways which may lead to untoward fibrosis, remain to be better clarified.

There are various histological definitions of granulomatous inflammation and different classification schemes (Adams 1976; Warren 1976; Williams & Williams 1983; Sheffield 1990). Nevertheless, most investigators would agree that granulomas reflect a chronic rather than acute form of inflammation, neutrophils are absent or not histologically prominent, and that mononuclear phagocytes (primarily resident macrophages) predominate and form the multinucleated core of these focal collections of aggregated cells. In immunologically active (epithelioid-type) granulomas, as opposed to foreign body-type granulomas, additional critical cells (e.g. antigen-presenting dendritic cells, cytokine-secreting T cells) are required for functional antimicrobial action. These cells are recruited along with immigrant blood monocytes and take up residence closely adjacent to or in the mononuclear cell mantle which slowly evolves to surround and/or infiltrate the core of infected macrophages. In some instances, B cells, plasma cells and eosinophils are also attracted.

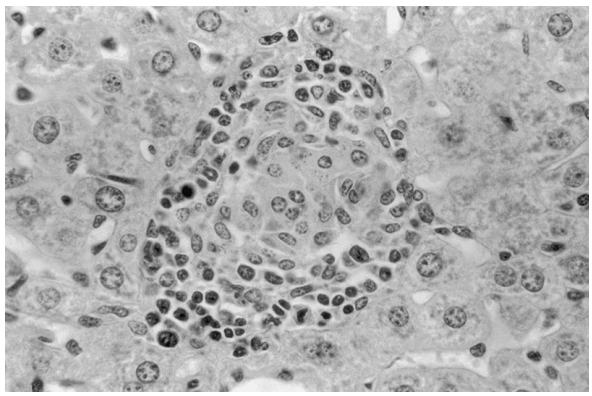

Figure 2. Mature liver granuloma 4 weeks after *L. donovani* infection in normal BALB/c mouse. Well-developed mononuclear cell mantle surrounds Kupffer cell core containing few residual amastigotes. ($\times$ 315). Reproduced with permission (Squires *et al*. 1989).

Thus, the structure of the mature tissue granuloma induced by a representative intracellular infection (in the case of this report, experimental visceral leishmaniasis caused by *L. donovani*) consists of a core of fused, parasitized resident macrophages with an encircling mononuclear cell mantle containing blood monocytes and both CD4 + and CD8 + T cells (Murray 1999). Figure 2 illustrates these features in a granuloma provoked by parasitized Kupffer cells in the liver of BALB/c mice infected four weeks previously with *L. donovani*. While macrophages in the spleen and bone marrow are also targeted in visceral leishmanaisis, the liver is the best-studied organ in mouse models of *L. donovani* infection (Baker *et al*. 1999; Murray 1999).

Granuloma structure and function in experimental visceral leishmaniasis

Two recent reviews of granulomatous inflammation in visceral infection in mice (Baker *et al*. 1999; Murray 1999) have already summarized the basic T (Th1) cell-dependent immunology of host defence against *L. donovani,* indicating how these responses interdigitate closely with or overlap the mechanisms involved in granuloma formation. These and other experimental findings (Gutierrez *et al*. 1984; Wilson *et al*. 1987; Laurenti *et al*. 1990; Correa *et al*. 1992; Wilson *et al*. 1996) have also been correlated with clinical observations

Table 1. Summary of liver granuloma structure-function relationships in experimental visceral leishmaniais*

	Structure	Function	Mice or experimental conditions
1.	Normal	Normal	Normal control BALB/c and C57BL/6
2.	None	None	T cell-deficient nude and CD4 or CD8 cell-depleted normals
			Monocyte influx-deficient (anti-CR3-treated normals and ICAM-1 gene-disrupted)
			Th1 cell cytokine-deficient (IL-12 and IFN-γ knockouts (KOs)**
			Induced Th2 cell-type response (IL-4/IL-10 in concert) in normals
			High-dose TNF treatment in normals
3.	Normal	None	Macrophage mechanism-deficient (iNOS KO)
			Th1 cell response-and macrophage-deactivated (IL-10 transgenic)
4.	Enhanced	Enhanced	Cytokine-treated normals (IL-2, GM-CSF)
			Absent IL-10 or IL-10 receptor blockade (IL-10 KO or anti-IL-10R-treated normals)
			Anti-CTLA-4-treated normals
5.	Malignant	Obscured	Absent TNF (TNF KO)**
6.	Enhanced	Normal	Cytokine-treated normals (IL-1, G-CSF)
			Normal enhanced cytokine-treated normals (IL-12, IFN-γ)
7.	None	Enhanced	Chronically infected (immune) normals
			Rechallenged immune mice treated with cyclosporin A

*See text for abbreviations. Normal granuloma structure = well-formed, mature granuloma (see Fig. 2). Normal granuloma function = reduction (killing) of amastigote burden and parasite-free granulomas at majority of infected foci.

**See text for discussion of effects of anticytokine mAbs directed at IL-2, IFN-γ, TNF and GM-CSF.

generated from patients with visceral leishmaniasis (kala-azar) (Murray 1999). However, relatively little pathological data regarding the tissue granuloma in human infection is actually available (Daneshbod 1972; Pampiglione *et al*. 1974a; Pampiglione *et al*. 1974b; Veress *et al*. 1977; Moreno *et al*. 1988; El Hag *et al*. 1994) since the typical clinical samples used in diagnosis (tissue imprints, smears of organ aspirates) yield little specific histological information.

The present report deals with experimental *L. donovani* infection in the liver, and focuses on the remarkably broad spectrum of granuloma structure-function relationships which can be uncovered in this model of intracelluar disease. Utilizing a wide range of experimental hosts and various exogenous treatments or manipulations of the immune response, seven reasonably distinct relationships can be identified (Table 1).

Relationship 1: normal structure-normal function – the basic model of granuloma assembly and acquired resistance in intact mice

Acquired resistance. Normal BALB/c and C57BL/6 mice, initially susceptible to *L. donovani*, permit rapid increases in intracellular liver parasite burdens during the first two to four weeks after intravenous (IV) challenge (Figure 3a). After weeks 3–4, however, acquired resistance develops and liver burdens plateau and decline; the self-curing phenotype in both strains of mice is then fully expressed as infection and driven to low levels by week 8. Although normal mice remain chronically infected, spontaneous relapse does not occur, and these animals are solidly resistant (immune) to subsequent rechallenge (Murray 1999).

Humoral immunity (specific antibody) plays little or no role in the preceding acquired resistance response. Instead, as shown in Table 2 (where the (0) symbol indicates the failure to assemble granulomas or control *L. donovani*), initially required host defence components are: (a) T cells (CD4 and CD8 cells) (Stern *et al*. 1988), (b) Mac-1 + influxing blood monocytes and intracellular adhesion molecule-1 (ICAM-1) expression (Cervia *et al*. 1993; Murray 2000b), (c) multiple interdigitating inflammatory cytokines, primarily of the Th1 cell-type (Murray 1999; Murray 2001) and (d) induction of microbicidal mechanisms in immigrant monocytes and resident macrophages (Murray & Nathan 1999). The key endogenous cytokines thus far identified in the model are interleukin 12 (IL-12), IL-2, IFN-γ, TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Murray *et al*. 1993; Tumang *et al*. 1994; Murray *et al*. 1995; Murray 1997; Taylor & Murray 1997; Engwerda *et al*. 1998; Wilson *et al*. 1998; Murray *et al*. 2000; Murray *et al*. 2000a;b; Satoskar et al. 2000). Important cytokine-induced or enhanced events include: (a) IL-12- and IL-2-triggered IFN-γ production (b) TNF secretion, and (c) mononuclear phagocyte recruitment, activation by IFN-γ and TNF, and elaboration of leishmanicidal reactive oxygen (ROI) and nitrogen intermediates (RNI) (Baker *et al*. 1999; Murray 1999; Murray 2001). The critical leishmanicidal effector cell appears to be the activated influxing blood monocyte (Cervia *et al*. 1993; Murray 2000b). Figure 3 surveys representative outcomes of *L. donovani* infection in mice

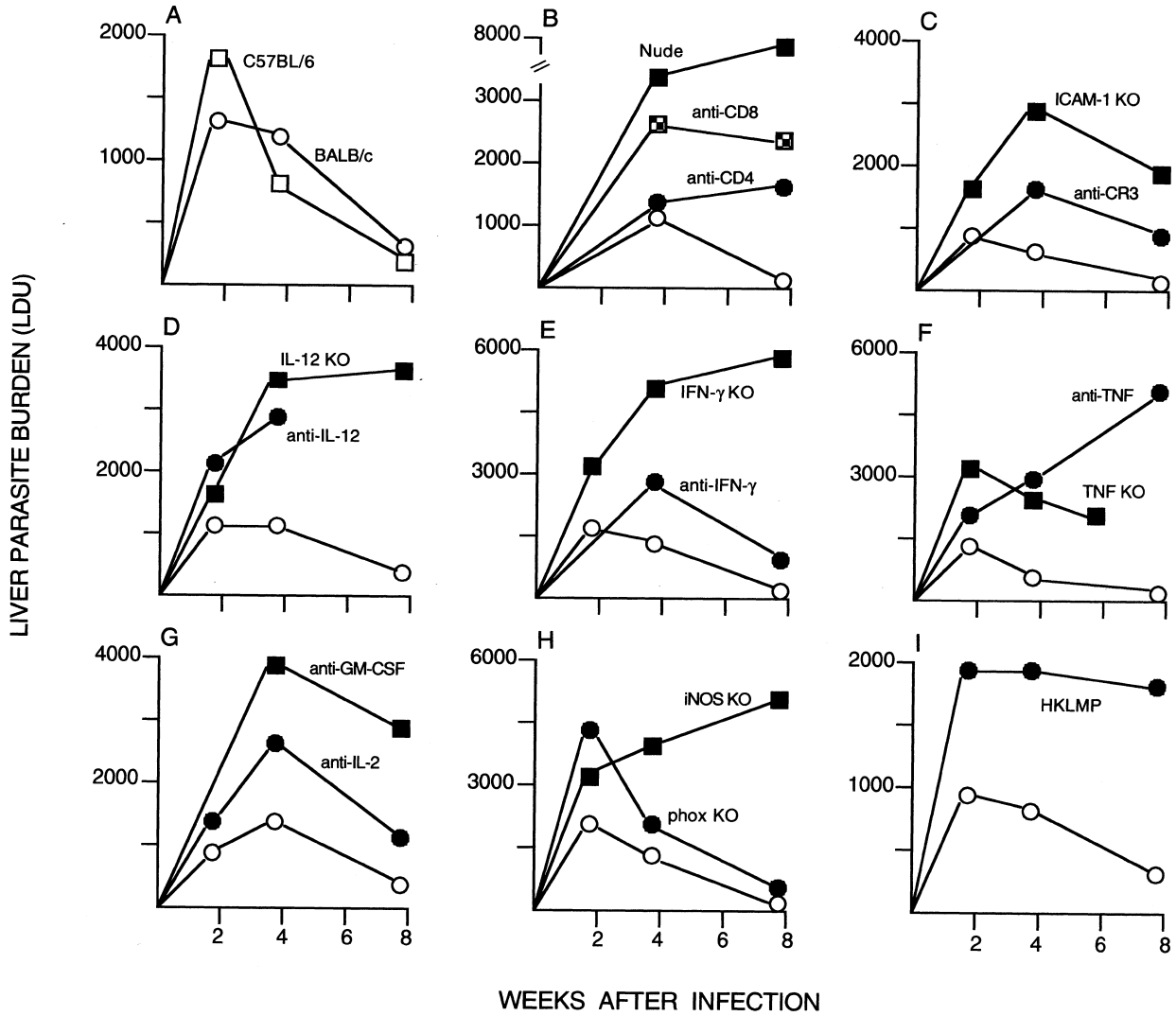

Figure 3. Course of *L. donovani* liver infection in normal mice (a), and in mice deficient in: (b) T cells or CD4 or CD8 cells (nude or CD4 or CD8 cell-depleted normal BALB/c mice) (c) influxing blood monocytes (ICAM-1 gene-disrupted and anti-CR3-treated normal BALB/c mice) (d-g) IL-12, IFN-γ, TNF, IL-2 or GM-CSF (cytokine gene knockout (KO) or anticytokine mAb-or antiserum-treated normal BALB/c mice) (h) macrophage leishmanicidial mechanisms (phox and iNOS KO mice), and (i) in mice expressing a Th2-type response (normal BALB/c mice preimmunized with heat-killed *L. major* promastigotes (HKLMP)). Open symbols in each panel indicate control animals. mAb or antiserum injections were begun 1 day before or 1 day after infection and were given up to thrice-weekly for 8 weeks. Note differences in vertical axis scales for mean liver parasite burdens (measured as Leishman-Donovan units (LDU)). (See text for references.)

deficient in one of the preceding cells, responses or mechanisms.

Experimental caveats. For two reasons, however, caution needs to be exercised in interpreting several of the results in Table 2 including those indicating mechanisms of granuloma assembly (see below).

First, different types of deficient mice were used in these experiments: normal mice treated with cytokine-depleting or -blockading monoclonal antibodies (mAb) vs. gene-disrupted (knockout (KO)) animals. In some instances, both types of mice were available for testing and therefore comparison. Results derived in mAb-treated mice are useful since acute depletion avoids the problem of atypical compensatory mechanisms which KO mice may be poised to express (or overexpress). At the same time, however, cytokine neutralizing activity may not satisfactorily reach the infected microenvironment and/or may also wane after repeated injections of anticytokine mAb (Squires *et al.* 1989). Such conditions

Table 2. Endogenous immune factors and mechanisms in acquired resistance and granuloma formation in experimental visceral leishmaniasis

Factor or mechanism	Deficient or Knockout (KO) host*	*Initial stage (weeks 2–4)* Acquired resistance	Granuloma assembly	*Late stage(weeks 4–8)* Resolution of infection	Mature granuloma
Normal mice					
BALB/c	–	+ + **	+ +	+ +	+ +
C57BL/6	–	+ +	+ +	+ +	+ +
Cells					
T cells	Athymic	0	0	0	0
CD4+	anti-CD4	0/+	0	0	0
CD8+	anti-CD8	0	0	0	0
NK#	antiasialo GM1	+ +	+ +	NT	NT
Monocytes	anti-CR3	0	0	+	+
	ICAM-1 KO	0	0	+	+
Cytokines					
IL-1	anti-IL1R	+ +	+ +	NT	NT
IL-12	anti-IL-12	0	0	NT	NT
	KO	0	0	0	
IL-2	anti-IL-2	0	0	+	+
IFN-γ	anti-IFN-γ	0	0	+	+
	KO	0	0	0	0
TNF	anti-TNF	0	+ + ##	0	+ + ##
	KO	0/+	0/+	***	***
GM-CSF	anti-GM-CSF	0	+ + ##	+	+ +
Macrophage Mechanisms					
Phox	KO	0/++	0/+	+ +	+ +
iNOS	KO	0	0/+	0	+ + ##

*All mAb-or antiserum-treated animals were normal BALB/c mice; all deficient or knockout (KO) mice were on a C57BL/6 background except for athymic (nude) and IL-12 KO mice (BALB/c).

**Symbols: (+ +), intact response; (+), clear response but not normal; (0), response absent; and (0/+) or (0/++), no response at week 2 but some (0/+) or intact response (0/++) at week 4.

#Abbreviations: NK, natural killer cells; NT, not tested; phox, phagocyte oxidase; iNOS, inducible nitric oxide synthase.

***See text. TNF KO mice show some antileishmanial activity and early granuloma formation at week 3; however, these responses are then obscured by uncontrolled inflammation and most mice are dead by week 8.

##Clear-cut dissociation between structure (intact, mature granulomas) and function (no antileishmanial activity). See text.

provide the setting for potentially misleading conclusions that an initially active, required factor had evolved to become dispensible and/or that its effects were compensated for by a separate, late-onset mechanism.

For instance, injecting normal BALB/c mice twice-weekly with anti-IFN-γ starting one day before *L. donovani* infection abolished acquired resistance and hepatic granuloma development up to week 4 (Figure 3e) (Squires *et al*. 1989), clearly demonstrating the cytokine's required role. Sinusoidal class II MHC expression (a likely surrogate marker for IFN-γ effect) was also completely suppressed by anti-IFN-γ treatment; however, class II MHC expression within the granuloma itself was unaffected. In addition, despite continued anti-IFN-γ injections, responses emerged by week 8, suggesting more slowly developing IFN-γ-independent pathway(s) for both antileishmanial activity and granuloma induction. However, by week 8, neutralizing anti-IFN-γ activity in the serum was considerably reduced in these chronically infected mice (Squires *et al*. 1989), pointing to incomplete IFN-γ inhibition rather than a separate mechanism at this late time point. Suboptimal cytokine neutralization in mAb-treated mice (which may also be germane to late emergence of function despite anti-IL-2 treatment (Murray *et al*. 1993) (Figure 3g and Table 2)), was subsequently supported by results in animals devoid of IFN-γ. In direct contrast to anti-IFN-γ-treated animals, IFN-γ KO mice developed neither resistance (Figure 3g) nor granulomas during the same eight-week period (Taylor & Murray 1997).

Irrespective of the effects of anticytokine mAb treatment, if antimicrobial activity and/or granuloma assembly are absent in KO animals devoid of the cytokine, it seems safe to conclude that: (a) the cytokine is indeed required and, parenthetically; (b) that a substitute mechanism is not available to the host. Conversely, if an infected cytokine KO mouse displays intact granuloma assembly, for example, two interpretations remain and may be difficult to distinguish: the cytokine is truly dispensible in the tissue reaction or a compensatory

mechanism has supervened. Neverthless, if compensatory effects develop late in the course of infection, accurate conclusions can still be drawn about the basis of early events. In addition, even if not ordinarily expressed in normal animals, identifying compensatory antimicrobial or granuloma-inducing mechanisms serves to expand the knowledge of the auxillary pathways of the host immune response.

Second, it should also be noted that the results in Table 2 are from experiments in which the state of acquired resistance and granuloma maturation in deficient mice was examined twice; first, during the early stage of visceral infection (weeks 2–4), and then at the time of expected resolution (week 8). As in all biological models of an immunological response, the role of a particular cell, cytokine or macrophage-specific mechanism may vary and assume greater or lesser importance depending upon the stage of infection. Thus, the data in Table 2 indicate the phase of visceral infection from which the results were obtained as well as whether mAb-treated normal and/or KO mice were tested.

Granuloma assembly. Figure 2 illustrates the histological appearance of the granuloma generated in the *L. donovani*-infected liver of normal BALB/c mice four weeks after infection, at the time acquired resistance is reaching expression. The tissue structure shown in Figure 2 is designated as both mature (well-developed mononuclear cell mantle surrounding a core of fused parasitized Kupffer cells) and functional (parasite-free) (Murray 1999). The results in Table 2 and Figures 4 and 5 show the required components, cellular composition and kinetics and histology of the response which converts the initially parasitized, isolated Kupffer cell to the mature functional granuloma. By week 8, granuloma disassembly (involution) is underway in normal mice and completed by week 12, at which time hepatic tissue has returned to an essentially undisturbed histological state (not shown) (Murray 1999).

The immunological mechanisms of granuloma assembly in normal mice during the first two to four weeks after *L. donovani* challenge are similar but not entirely superimposable on those implicated in initial acquired resistance (Table 2). The apparent exceptions relate to GM-CSF and TNF since repeatedly injecting normal mice with anti-GM-CSF or anti-TNF (starting one day after infection (Figure 3f,g)) impaired resistance but not early granuloma formation (Tumang *et al*. 1994; Murray *et al*. 1995). However, confirmatory testing in GM-CSF KO mice has not been performed, and in addition, the early dissociation between antileishmanial activity and granuloma development did not persist into the late stage of infection. By week 8, both activities emerged together in the face of continued anti-GM-CSF injections (with readily detected levels of circulating GM-CSF neutralizing activity), yielding an appropriate net response of resolution of infection within mature granulomas. This net structure-function relationship, measured at the time of anticipated resolution of visceral infection (week 8), is probably the most relevant to consider.

In anti-TNF-treated normal mice, the granulomatous tissue reaction was also preserved despite clear-cut inhibition of acquired resistance (Figure 3f) (Tumang *et al*. 1994). However, in TNF KO mice, early granuloma assembly at week 2 was entirely absent, correlating with particularly high liver parasite burdens at this time point (Murray *et al*. 2000a) and leading to the more reasonable conclusion that TNF is indeed an initially important granuloma-inducing factor. Results in other models have similarly identified TNF as a central modulator of chemokine responses and recruitment of T cells and monocytes (Saunders & Cooper 2000). As also discussed below, the subsequent course of the hyperinflammatory tissue response in *L. donovani*-infected TNF KO mice was remarkably aberrant, re-emphasizing TNF's less well-appreciated anti-inflammatory action (Marino *et al*. 1997; Murray *et al*. 2000a).

Observations made during the resolution stage of infection after week 4 also yielded an additional example of apparent dissociation between control of infection and granuloma development in anticytokine mAb-treated vs. cytokine KO mice, in this instance for IFN-γ (discussed above) Although anti-IFN-γ-treated mice showed late granuloma development, the more relevant observation, pointing to this cytokine's persistent required role, was that IFN-γ KO mice showed none (Taylor & Murray 1997; Murray & Delph-Etienne 2000). It is important to note, however, that there were reasonably concordant results in these studies for mAb-injected vs. KO animals as related, for example, to IL-12 in the initial stage of infection (Murray 1997; Murray *et al*. 2000; 2000b) (Table 2). Further, normal mice rendered deficient in blood monocyte influx by mAb-induced blockade of type 3 complement receptor (CR3, Mac-1) (Murray 2000b) and gene-disrupted mice deficient in expression of the adhesion molecule ICAM-1 (the principal endothelial cell ligand for monocyte CR3) also behaved similarly (Murray 2000b). In both groups of animals, *L. donovani*-induced monocyte influx and granuloma assembly failed to develop initially but began to emerge after week 4 via a late-acting, apparent CR3-and ICAM-1-independent compensatory pathway. This latter pathway was considerably better expressed in ICAM-1-deficient mice

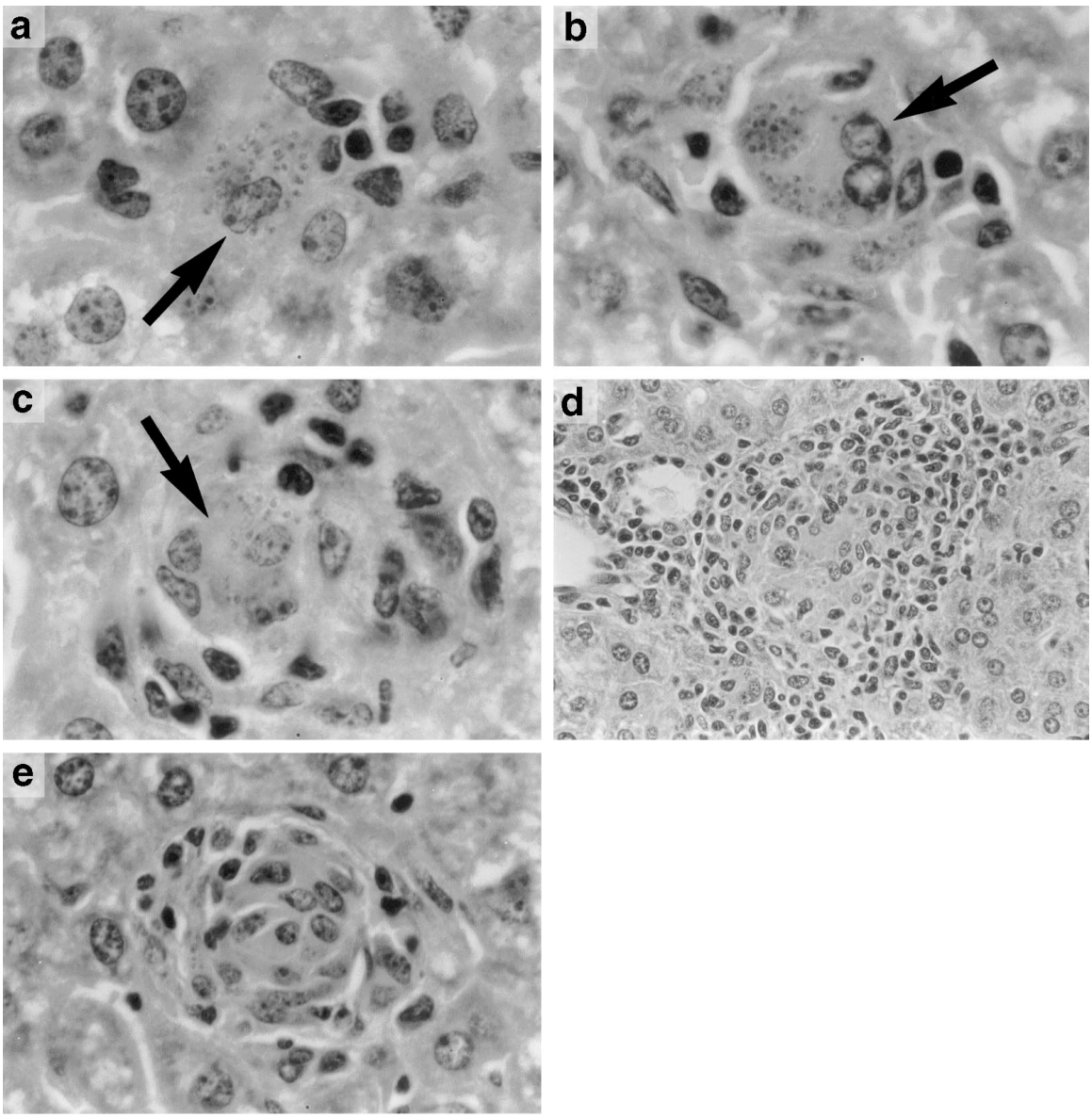

Figure 4. Granuloma histological evolution in normal BALB/c mice 1–8 weeks after *L. donovani* infection. (a and b) Week 1. Initial parasitization of resident macrophages (Kupffer cells (KC)) (arrow), some KC fusion, and minimal mononuclear cell recruitment. (c) Week 2. Early granuloma assembly at fused parasitized KC (arrow) with initial attraction of influxing lymphocytes and monocytes. (d) Week 4. Mature, functional (parasite-free) granuloma (also see Figure 2). (e) Week 8. Involuting epithelioid granuloma devoid of amastigotes. By week 12 (not shown), tissue inflammation had nearly entirely resolved. Overall histological reaction in normal C57BL/6 mice was similar (see Figure 6C) but developed somewhat more rapidly. (a, b and c × 500; d × 315; and e × 400). (a, c and e from (Murray 1999); b from (Murray *et al*. 1987); and d from (Cervia *et al*. 1993); reproduced with permission).

vs. anti-CR3-injected normal animals, perhaps reflecting an adaptive response in a gene-disrupted host. In addition, in both types of monocyte influx-deficient mice, the state of granuloma development fairly correlated with the absence or presence of antileishmanial activity (Cervia *et al*. 1993; Murray 2000b) (Figure 3c and Table 2).

Relationship 2: no structure-no function

Deficient cells. In view of the basis of granuloma formation and the central role of T cells and monocytes in acquired resistance, it was entirely predictable that this relationship – no granuloma and no function (no anti leishmanial activity) – would be clearly displayed in liver tissue of T cell-deficient

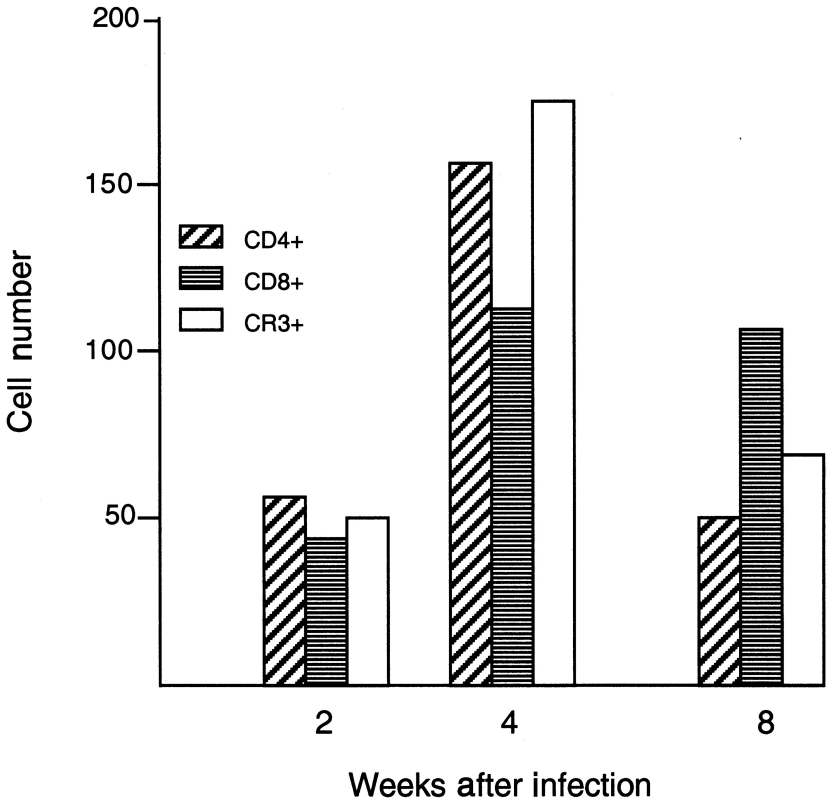

Figure 5. Kinetics of mononuclear cell influx into parasitized liver foci in *L. donovani*-infected BALB/c mice. Results (immunocytochemistry) indicate mean number of granuloma cells in 20 ×63 microscopic fields which expressed CD4, CD8 or CR3 (McElrath *et al*. 1988).

mice and in animals with defective blood monocyte influx (Figure 6). However, it is worth pointing out that one initial step in hepatic granuloma formation, Kupffer cell fusion, was preserved to varying degrees in both T cell-and monocyte-deficient mice (Murray 1999; Murray 2000b) (Figure 6) (as well as in all other deficient mice listed in Table 2). Thus, fusion of parasitized resident macrophages, forming the multinucleated granuloma core, is largely independent of the host immune response in this model. Nevertheless, lacking recruited mononuclear cells and showing no cellular mantle, such foci do not fulfil the criteria used in these studies for either early or mature granulomas (Murray 1999) and are designated here in the 'no structure' category.

The observations in T cell-deficient nude and normal BALB/c mice depleted of CD4 or CD8 cells by mAb injection indicated that both T cell subsets were required for successful granuloma assembly and function (Stern *et al*. 1988). This conclusion was confirmed in parallel experiments in which responses in nude mice were successfully reconstituted only if both CD4 and CD8 cells were transferred. Depleting normal BALB/c mice of natural killer cells by antiasialo-GM1 antiserum injections had no effect on either granuloma formation or acquired resistance (Murray 1999).

Inspection of parasitized foci in anti-CR3-treated normal mice and ICAM-1 gene-deficient animals showed few closely applied mononuclear cells of any cell type (Figure 6). Thus, the primary absence of infiltrating monocytes resulted in a paucity of lymphocytes, suggesting that monocyte-derived factors are among the signals which regulate T cell recruitment in the presence of intracellular *L. donovani*. Studies from the Kaye laboratory using this model have suggested, for example, possible early roles for several chemokines (macrophage inflammatory protein-1α, monocyte chemoattractant protein-1, interferon-γ-inducible protein-10 (IP-10)) and a more sustained role for IP-10 in enhancing attraction of T cells (Cotterell *et al*. 1999). The absence of any mononuclear cells (e.g. monocytes) at infected foci in nude mice (Figure 6) also conversely implicated T cell-derived factors in monocyte influx. One such factor, secreted by both CD4 and CD8 cells, is likely to be IFN-γ (Stern *et al*. 1988; Tannenbaum & Hamilton 2000). As discussed previously, a late-onset CR3- and ICAM-1-independent compensatory mechanism for monocyte influx and granuloma assembly subsequently develops and is associated with *L. donovani* killing (Murray 2000b).

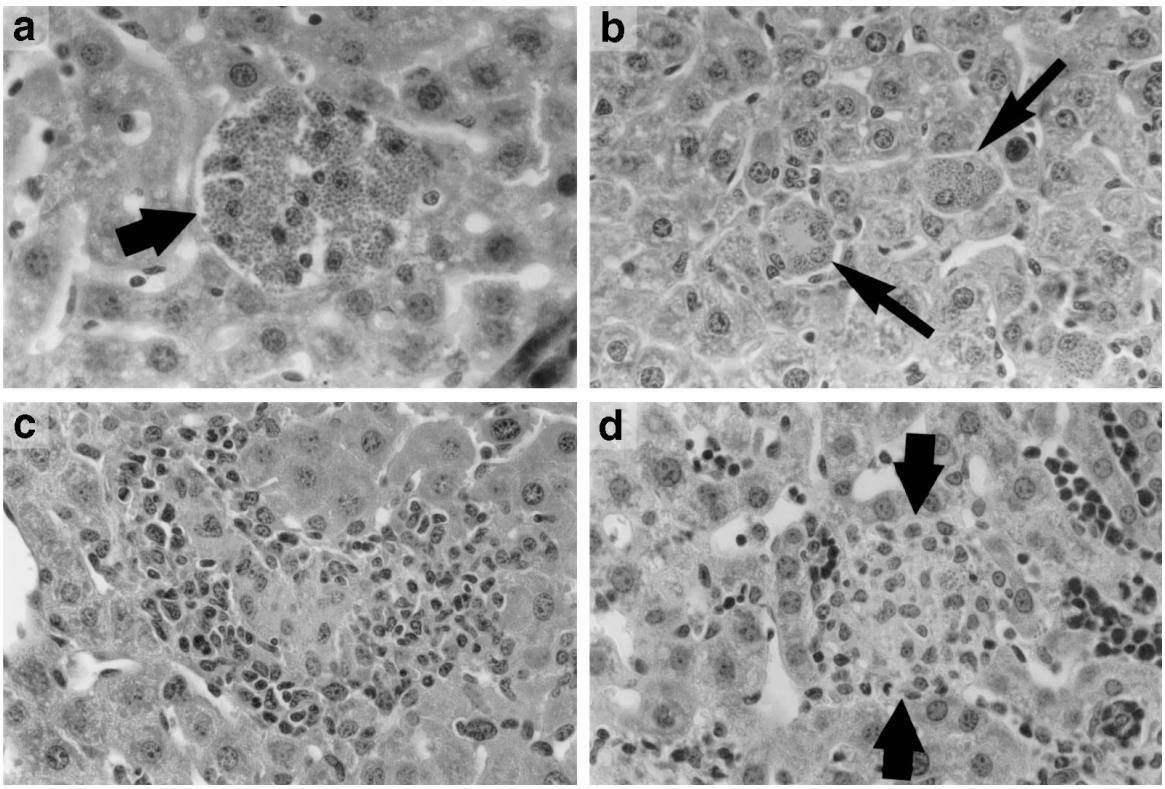

Figure 6. Liver histological reaction 4 weeks after *L. donovani* infection in mice deficient in T cells or influxing blood monocytes. (a) Athymic (nude) BALB/c mice show heavily parasitized, fused Kupffer cells and absence of surrounding mononuclear cells. (b) Blockade of monocyte influx by anti-CR3 treatment abolishes granuloma formation at parasitized foci (arrows) in normal BALB/c mice. Compare (a) and (b) to Figs 2 and 4(D). (c) Normal C57BL/6 mice show mature granuloma formation. (d) In contrast, in C57BL/6 ICAM-1-deficient mice, mononuclear cells remain largely within adjacent sinusoids and few cells surround or enter multinucleated infected foci (arrow). (a-d $\times$ 315). (A from (Murray *et al*. 1987); b from (Cervia *et al*. 1993); and c and d from (Murray, 2000b); reproduced with permission).

Deficient cytokines. In KO mice, the no structure-no function relationship was also expressed in the presence of deficient IL-12, IFN-γ and TNF (and in anti-IL-2-treated animals) in the early stage of visceral infection and, in IL-12 and IFN-γ KO mice, extended into the late stage (Table 2, Figures 3 and 7) (see Figure 10b for TNF KO mice). IFN-γ and TNF are clearly recognized signals for adhesion-related events, chemokine induction, T cell and monocyte recruitment to inflammatory sites, and monocyte/macrophage activation with induction of leishmanicidal mechanisms (Roach *et al*. 1991; Adams & Lloyd 1997; Luster 1998; Murray 1999; Saunders & Cooper 2000; Marquez & Martinez 2001). While IL-2 expands and mobilizes T cells, its primary role as well as that of IL-12 in this model may largely be the induction of IFN-γ and subsequent IFN-γ-mediated reactions (Murray *et al*. 1993). IL-12 is also much more broadly acting, however, and is thought to be the key initiator of the basic antileishmanial Th1 cell-associated

Table 3. Kinetics of the histological response to *L. donovani* Infection in the liver in normal BALB/c mice

Extent of infection and cellular response*	*Weeks after infection* 1	2	4	8
Liver parasite burden (LDU)	810	1822	1691	206
Infected foci (no.)	69	92	82	21
No cellular response (%)	92	26	6	1
Developing granuloma (%)	8	67	49	7
Mature granuloma (%)	0	7	36	34
Parasite-free granuloma (%)	0	0	9	58

*Liver imprints were used to determine amastigote burdens (expressed as Leishman-Donovan units (LDU), and the number of discrete infected foci per 50 $\times$ 63 microscopic fields was counted in histological sections (Murray 1999). Results are mean values. (Responses in normal C57BL/6 mice were similar, although expressed somewhat more rapidly.) The cellular reaction at each infected focus was scored as (a) no cellular response – isolated, unfused or fused parasitized Kupffer cells (KC) alone (b) developing granuloma – fused KC with some cellular infiltrate (c) mature granulomas – well-organized reaction with mononuclear cell mantle surrounding parasitized multinucleated core of KC, and (d) parasite-free – no visible amastigotes. See histological correlates in Fig. 4.

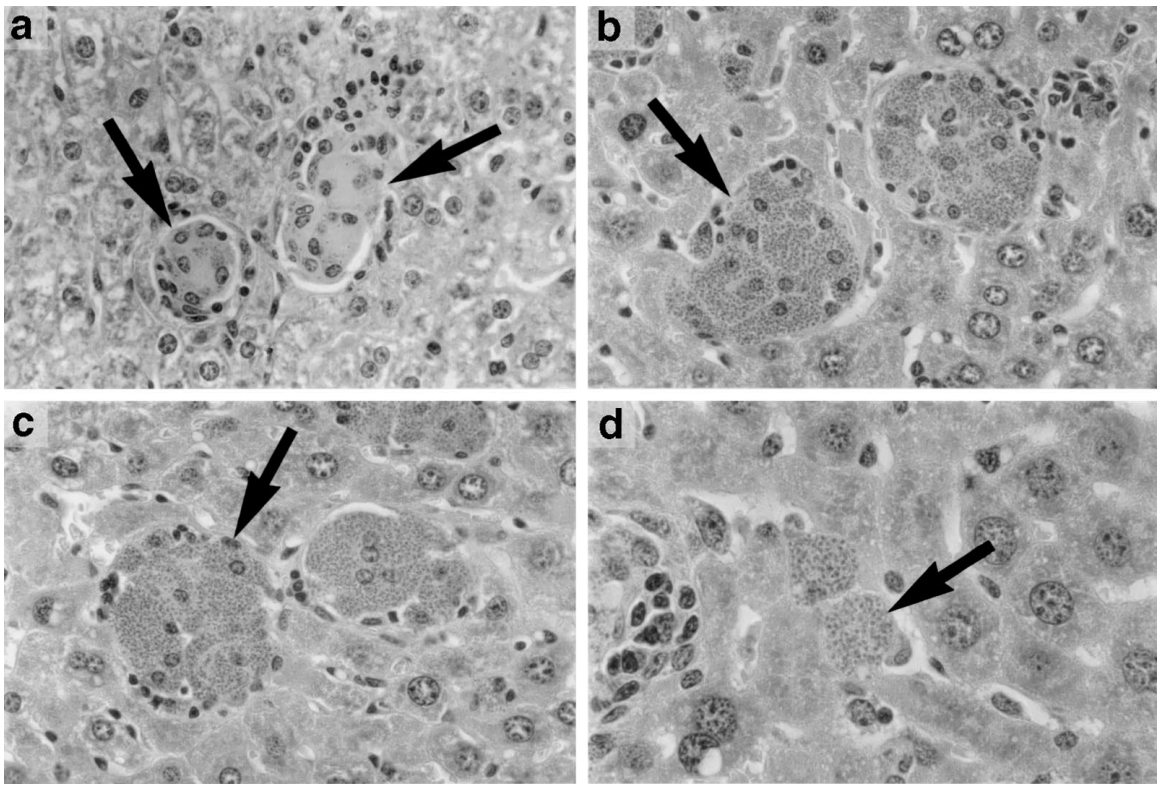

Figure 7. Suppression of liver granuloma assembly 4–8 weeks after infection in Th1 cell-associated cytokine-deficient mice and in mice induced to express a Th2 cell-type response. (Compare to Figure 4 for tissue reactions in control mice.) (a) Week 4. Normal BALB/c mice treated with anti-IL-2. (b and c) Week 8. BALB/c IL-12 knockout (KO) (b) and IFN-γ KO mice (c). (d) Week 4. Normal BALB/c mice induced by preimmunization with heat-killed *L. major* promastigotes to crossreact to *L. donovani* with a Th2-type (IL-4 plus IL-10) response. Except for strikingly heavier infection in IL-12 and IFN-γ KO mice, histological appearance in a-d shows similarly aborted granuloma assembly (arrows) with near-complete absence of recruited mononuclear cells in Th1 cell cytokine-impaired hosts. (a-c × 315; and d × 500). (a from (Murray *et al*. 1993) and b from (Murray *et al*. 2000b); with permission).

response (Engwerda *et al*. 1998; Murray *et al*. 2000b; Satoskar *et al*. 2000).

Deactivation. Despite the presence of appropriate cells and Th1 cell-type cytokines, the no structure-no function relationship can also be demonstrated in mice which display an additional immunodeactivating Th2 cell-associated response. In normal BALB/c mice, cutaneous infection with *L. major* spontaneously induces a polarized, IL-4-driven and highly suppressive reaction resulting in the noncure phenotype (Reiner & Locksley 1995). In contrast, the same BALB/c mice do not spontaneously show a functional Th2 cell-type reaction in response to *L. donovani* and must first be experimentally manipulated (Miralles *et al*. 1994; Murray *et al*. 1997). Thus, although mRNA expression for IL-4 and IL-10 (as well as IL-12, IL-2 and IFN-γ) is induced in normal BALB/c mice within the first one to two weeks after *L. donovani* challenge (Miralles *et al*. 1994; Engwerda

Table 4. Tissue effects induced by exogenous cytokine treatment*

Cytokine treatment in infected mice	Leishmanicidal activity	Granuloma modifying effect
IFN−γ	+ **	0
IL−12	+	0
TNF	Inhibited	Inhibited
IL−2	+	+ +
GM-CSF	+	+ +
G-CSF	0	+ +
IL-1	0	+

*Except for IL-1, treatment with listed cytokines was given to normal BALB/c mice starting 2 weeks after *L. donovani* infection; tissue effects were measured after 7days of treatment.

**Symbols: (0), no effect; (+) enhanced effect; and (+ +), striking effect.

#B10.D2/*n* mice, chronically infected for 32–36 weeks before IL-1 treatment (Curry and Kaye 1992).

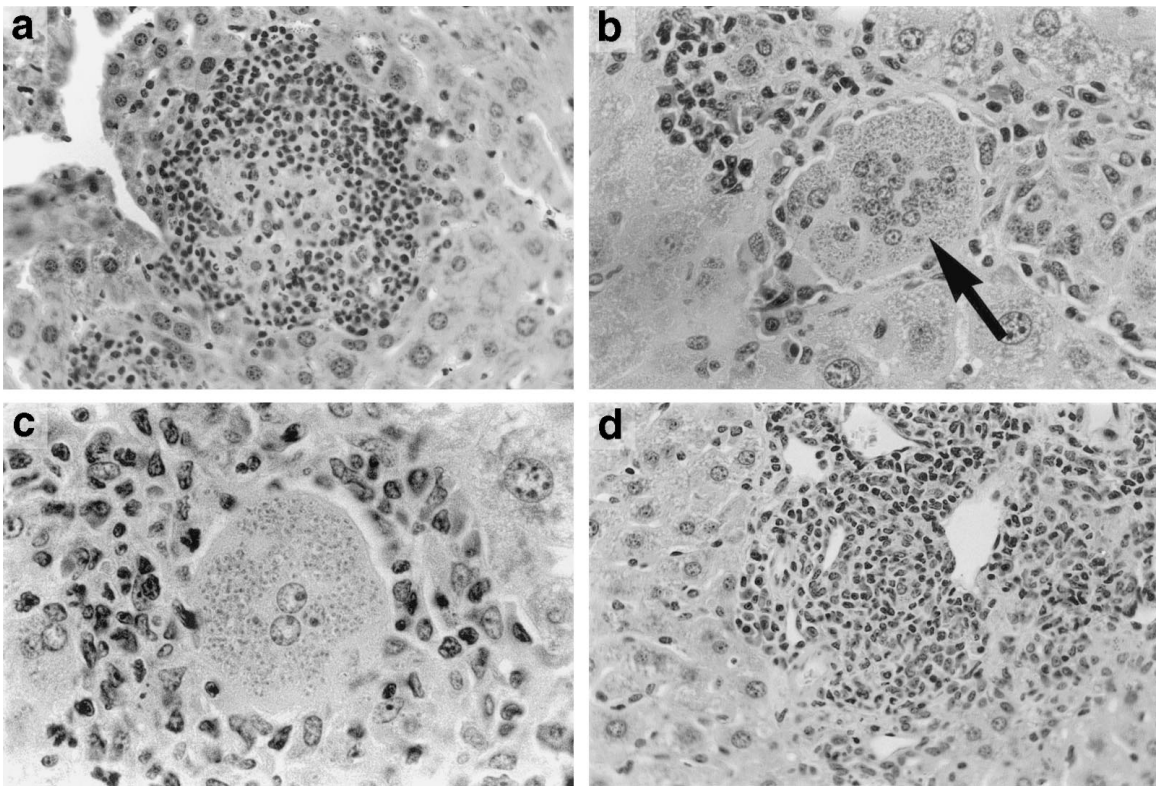

Figure 8. Intact granuloma assembly 4–8 weeks after infection in mice deficient in macrophage leishmanicidal mechanisms. (a and b) Week 4. (a) Well-established granuloma in C57BL/6 wild-type controls, and (b) developing but heavily infected granuloma (arrow) in C57BL/6 iNOS knockout (KO). (c and d) Week 8. (c) Granulomas in iNOS KO mice are mature and structurally intact but contain a striking load of intracellular amastigotes – defining the 'ineffective granuloma.' (d) Reaction is mature and intense in X-chronic granulomatous disease (C57BL/6 phox KO) mice and granulomas show few amastigotes. (a, b and d × 315; and c × 500). Reproduced from (Murray & Nathan 1999) with permission.

et al. 1996; Melby *et al*. 1998; Wilson *et al*. 1998; Cotterell *et al*. 1999), the IL-4/IL-10 Th2-type response is either insufficient or overshadowed since these animals proceed to develop Th1 cell-dependent acquired resistance, granuloma formation and the self-cure phenotype (Miralles *et al*. 1994; Murray *et al*. 1997).

This balance can be experimentally altered, however, by first immunizing BALB/c mice with a preparation of heat-killed *L. major* promastigotes. Upon subsequent challenge with viable *L. donovani*, immunized mice crossreact with an IL-4- and IL-10-dependent response which induces the noncure phenotype (Murray *et al*. 1997). In this setting, the well-studied, broad deactivating effects of both IL-4 and IL-10 on the Th1 cell response (Reiner & Locksley 1995; Spellberg & Edwards 2001) are expressed as inhibition of mononuclear cell influx and granuloma assembly, significantly impaired granuloma maturation, high liver parasite burdens and failure to resolve infection (Figures 3i and 7). IL-4 and IL-10 act in concert in this model since prophylactic treatment of immunized mice with either anti-IL-4 or anti-IL-10 reverses deactivation and restores the normal granuloma structure-function relationship (Murray *et al*. 1997). IL-13 and transforming growth factor-β (TGF-β) have also been implicated in suppression of antileishmanial resistance (Wilson *et al*. 1996; Wilson *et al*. 1998; Babaloo *et al*. 2001); therefore, both of these cytokines might also influence granuloma formation.

Excess cytokine (TNF). Five cytokines have been identified in this *L. donovani* model as required, pro-host defense endogenous factors (IL-12, IL-2, IFN-γ, GM-CSF, TNF) (Table 2) (Baker *et al*. 1999; Murray 1999; Satoskar *et al*. 2000; Murray 2001). In infected normal BALB/c mice, exogenous administration of these cytokines in recombinant form produces beneficial effects on granuloma assembly and/or antileishmanial activity with one exception, TNF (Tumang *et al*. 1994; Murray 1999) (Table 4). (The actions of exogenous IL-12, IL-2, IFN-γ and GM-CSF are considered elsewhere

according to their effects on granuloma structure.) In two-week infected mice, high-dose TNF (10 μg/day), administered continuously by pump for seven days, was given in an attempt to *enhance* macrophage activation and antileishmanial activity since TNF is involved in both of these linked events (Roach *et al*. 1991; Tumang *et al*. 1994; Murray *et al*. 2000a). However, just the opposite effect was induced as TNF treatment brought the progress of liver granuloma assembly to a standstill and exacerbated infection, yielding the no structure-no function relationship (Tumang *et al*. 1994). The mechanisms of this particular antigranuloma effect have not been examined. However, it is important to point out that a certain level of endogenous TNF is still strictly required not only for granuloma formation and acquired resistance, but also as judged by outcome in TNF KO mice (see below), to paradoxically limit the intensity of the Th1 cell cytokine response and protect infected tissues (Murray *et al*. 2000a).

Relationship 3: normal structure-no function – the ineffective granuloma

Deficient macrophage mechanisms. The notion that a structurally intact-appearing granuloma necessarily correlates with intact function was dispelled by observations in mice in which the critical tissue effector cell, the mononuclear phagocyte, was either deficient in a required leishmanicidal mechanism (Table 2) or was deactivated. Thus, after an initial delay following *L. donovani* challenge, inducible nitric oxide synthase (iNOS) KO mice (incapable of generating macrophage-derived RNI) developed abundant granulomas in the liver with the histological hallmarks of mature structures (Figure 8). Nonetheless, eight weeks after infection, fully formed granulomas in iNOS KO animals continued to expand and mature while remaining packed with amastigotes, testifying to ineffective function in the absence of a specific mononuclear phagocyte leishmanicidal mechanism (Figures 3h and 8) (Murray & Nathan 1999). Macrophages and monocytes also kill *L. donovani* by generating ROI via the action of phagocyte NADPH oxidase (phox). After a similarly brief period of expressing the no structure-no function relationship, phox-deficient (X-chronic granulomatous disease) mice also readily assembled mature functional granulomas (Figure 8), a portion of which were exuberant and persistent. However, and in direct contrast to iNOS KOs, phox-deficient mice established intact structure-function conditions and controlled visceral infection after week 2 in an entirely normal manner (Table 2 and Figure 3h) (Murray & Nathan 1999).

Macrophage deactivation. Preliminary results indicate that the normal structure-no function relationship can also be induced if mononuclear phagocytes within the mature granuloma are deactivated by the sustained presence of the suppressive cytokine, IL-10 (H. Murray. Unpublished observations). Thus, *L. donovani*-infected IL-10 transgenic BALB/c mice eventually generate structurally intact hepatic granulomas but visceral infection is overtly uncontrolled, defining these tissue structures as ineffective. iNOS induction is one of the targets for IL-10's suppressive action (Groux *et al*. 1999), and it probably is not coincidental that the well-formed but heavily parasitized granulomas in eight-week infected IL-10 transgenic mice resemble those in iNOS KO mice shown in Figure 8. IL-10 is thought capable of globally disabling Th1 cells and inhibiting effector cell responsiveness to Th1 cytokines (Groux *et al*. 1999; Spellberg & Edwards 2001) – the same overall mechanism which mediates granuloma assembly and antileishmanial activity in this model. Thus, the normal structure-no function relationship in the presence of sustained IL-10 raises additional basic questions, for example, about (a) differential resistance of granuloma formation vs. antileishmanial acivity to IL-10 and/or (b) possible thresholds for Th1 cell-dependent responses above or below which a particular response is or is not expressed.

Relationship 4: enhanced structure-enhanced function

Two manipulations, administration or removal of a single selected cytokine, can modify the granuloma resulting in enhanced antileishmanial activity (Table 4). A third intervention, directed at enhancing CD28-mediated T cell costimulation pathways and therefore Th1 cell cytokine secretion (Murphy *et al*. 1998), can achieve a similar effect. Defined arbitrarily, granuloma modification reflects changes including increased number or size and cellularity (hypertrophy), cellular remodeling (new predominant cell) and/or accelerated kinetics of assembly.

Exogenous cytokine administration. In normal BALB/c mice, the developing granuloma can be strikingly modified by exogenous administration of either IL-2 or GM-CSF (Murray *et al*. 1993; Murray *et al*. 1995). Figure 9 illustrates the effects of seven days of continuous cytokine treatment by subcutaneous pump implanted two weeks after *L. donovani* infection. Livers of mice treated with human IL-2 showed marked focal accumulation of mononuclear cells, mostly lymphocytes; treatment with murine GM-CSF induced the appearance of large numbers of myelomonocytic cells (neutrophils

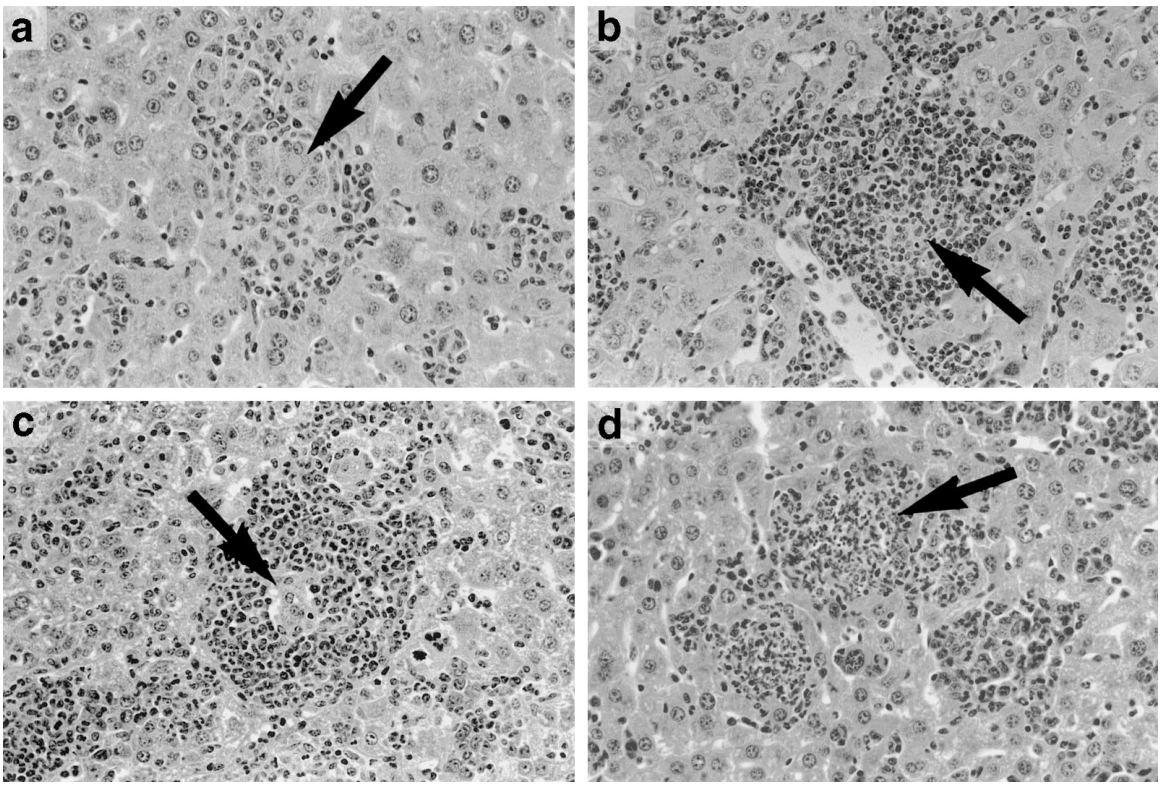

Figure 9. Modification of developing liver granulomas in normal BALB/c mice by exogenous cytokines. Starting two weeks after *L. donovani* infection, saline or cytokine treatment was administered continuously for 7 days by subcutaneous pump. (a-d) 3-week infected mice, treated for the previous 7 days with (a) saline (control) (b) human IL-2 (c) murine GM-CSF or (d) human G-CSF, show striking accumulations of mononuclear cells (IL-2), myelomonocytic cells (GM-CSF) and granulocytes (G-CSF), respectively, which encase parasitized foci (arrows). ($\times$ 200) (a from (Tumang *et al*. 1994); b from (Murray *et al*. 1993); and c and d from (Murray *et al*. 1995); reproduced with permission).

and monocytes). In both instances, influxing cells were preferentially drawn to infected tissue sites, enlarged the developing granuloma and literally encased parasitized Kupffer cells. In addition, the modifications induced by IL-2 and GM-CSF were also expressed by increased control over infection (enhanced function) (Table 4) (Murray *et al*. 1993; Murray *et al*. 1995). Granuloma-deficient nude BALB/c mice were also treated with IL-2 or GM-CSF in an attempt to stimulate similar effects. However, influx of cytokine-mobilized cells from peripheral blood to infected tissue proved to be strictly T cell-dependent; nude mice failed to show either granuloma assembly or antileishmanial activity in response to IL-2 or GM-CSF (Murray *et al*. 1993; Murray *et al*. 1995).

Endogenous cytokine removal. Preliminary experiments suggest that the absence of IL-10 (or blockade of its receptor) also effectively induces the enhanced structure-enhanced function relationship (H. Murray. Unpublished observations). Two weeks after infection, granuloma development is just underway in normal BALB/c animals and acquired resistance is not yet in evidence. However, at the same time point in BALB/c IL-10 KO mice, both basic responses are clearly accelerated, particularly granuloma assembly which is near fully expressed. In comparison to normal mice, granulomas in livers of two-week infected KOs are three times more numerous, considerably larger and more cellular, and the majority are already mature in appearance or function (parasite-free). Visceral infection in KO mice then proceeds to near-resolution as early as week 3, an outcome not seen in normal mice until eight weeks after infection. By week 4, when granuloma formation is still peaking in normal controls, the initially exuberant tissue reaction in IL-10 KOs is appropriately involuting, having already achieved its full host defence effect. Results from other models have consistently shown that IL-10 KO mice are poised to react to microbial challenge with an intense Th1 cell cytokine response and production of increased levels of IL-12 and IFN-γ (Gazzinelli *et al*.

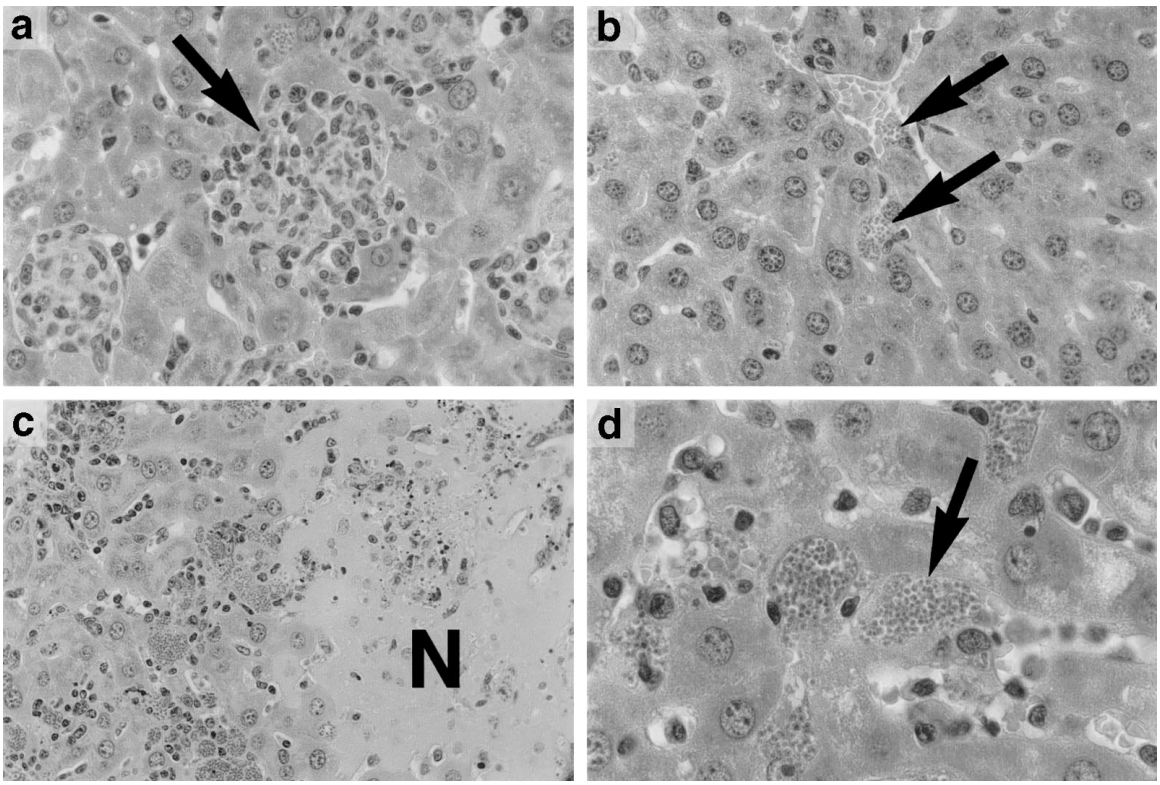

Figure 10. Liver histological reaction to *L. donovani* in C57BL/6 TNF knockout (KO) mice. (a and b) Week 2. (a) Normal, early granuloma assembly at infected Kupffer cells in C57BL/6 wild-type controls, but (b) none at parasitized foci in TNF KO mice (arrows). (c-d) Week 4. KO mice transiently express early granuloma formation at week 3 (not shown), but by week 4 (c), the accelerated hyper-inflammatory response is overtly destructive, leaving widespread hepatic necrosis (N), cell debris and few recognizable granulomas. Necrotic areas in (c) are ringed by partially preserved tissue containing large masses of intracellular amastigotes (arrows) (d). (a and b × 315; c × 200; and d × 500.) Reproduced with permission from (Murray *et al.* 2000a).

1996), the two key activating and granuloma-inducing cytokines in this *L. donovani* system.

Although IL-10 is induced by week 2 in normal BALB/c mice challenged with *L. donovani*, these animals control infection after week 4, leading to a prior conclusion that, overall, IL-10 (as well as coinduced IL-4) plays little functional (suppressive) role in this model (Miralles *et al.* 1994; Engwerda *et al.* 1996; Engwerda *et al.* 1998; Melby *et al.* 1998; Cotterell *et al.* 1999). However, this conclusion should be revised. Injecting normal BALB/c mice once-weekly with anti-IL-10 receptor mAb, starting one day after infection, converts these mice to essentially the same phenotype as IL-10 KOs – granuloma number is doubled and granuloma assembly and maturation and all phases of antileishmanial activity (initial control over parasite replication, onset of parasite killing and resolution) are markedly accelerated (H. Murray. Unpublished). Similar treatment in normal BALB/c mice with anti-IL-4 mAb has no effect on either activity, consistent with prior evidence indicating that endogenous IL-4 is probably not a determinant of outcome in models of experimental visceral infection (Miralles *et al.* 1994; Murray *et al.* 1997; Cotterell *et al.* 1999). Thus, release and expansion of the Th1 cell-associated response, induced by inhibiting IL-10 s effects by receptor blockade, may represent a new, logical form of granuloma-enhancing antileishmanial immunotherapy.

T cell costimulation. Optimal T cell activation, which forms the basis of proceeding to both acquired resistance to *L. donovani* and granuloma formation, is dependent upon costimulatory signals provided by antigen presenting cells. In contrast to CD28, T cell-expressed CTLA-4 can transduce negative, downregulating signals in the CD28/CTLA-4:B7 pathway, resulting in suppression of CD28-mediated Th1 and/or preferential induction of Th2 cell-type responses (Bluestone 1997). By blocking CTLA-4 in *L. donovani*-infected BALB/c mice by anti-CTLA-4 injections, Murphy *et al.* (1998) demonstrated enhanced IFN-γ expression, induction of IP-10, accelerated control of

visceral infection, and strikingly increased granuloma formation and maturation. While CTLA-4 therefore represents a legitimate target for immunoblockade and strengthening of the granuloma structure-function relationship, additional findings in experimental infection also point to TGF-β as a mediator of CTLA-4-induced downregulation (Gomes *et al*. 2000). Thus, neutralizing TGF-β, which has already been implicated in inhibition of granuloma T cell responses in this model (Wilson *et al*. 1998), may represent an alternative approach to enhancing host defence and the granulomatous tissue response in parasitized mice.

Relationship 5: malignant structure-obscured function – the special case of TNF KO mice

The consequences of an unregulated granulomatous reaction in the *L. donovani* model are well-illustrated in TNF KO mice. Two weeks after infection, KOs show no early granuloma assembly and permit high-level intracellular parasite replication in liver macrophages (Table 2 and Figures 3f, 8, and 10)), consistent with an initially required role for endogenous TNF (Murray *et al*. 2000a). One week later, however, these animals begin to show granuloma development. Nevertheless, at week 4, the inflammatory reaction abruptly accelerates, mononuclear cells pour into the liver in an uncontrolled fashion and wide-spread tissue necrosis replaces early granuloma formation (Figure 10). Although parasite burdens plateau at week 4 and then decline somewhat, additional effects are obscured since KO mice begin to die by week 6 and 100% are dead by weeks 8–10 with diffuse hepatic necrosis (Murray *et al*. 2000a). These observations are similar to those reported in different models in which TNF or TNF receptor KO mice were challenged with other pathogens or even nonviable microbial stimuli (reviewed in Murray *et al*. 2000a). Together, these findings, which re-emphasize TNF's anti-inflammatory action, clearly attest to the malignant consequences of a poised-to-erupt and subsequently unrestrained Th1 cell-type response in the tissues (Marino *et al*. 1997). While diffuse and fatal, the hyper-inflammatory reaction induced by *L. donovani* in the absence of TNF is not necessarily a runaway phenomenon. If two-week infected TNF KOs are treated with amphotericin B, rapidly eliminating most parasites, the aberrant tissue response is aborted and long-term survival increases from 0 to 88% (Murray *et al*. 2000a).

Relationship 6: enhanced structure-normal function and normal structure-enhanced function – additional cytokine-induced effects

There are three experimental settings, each specifically related to treating parasitized mice with exogenous cytokine, which demonstrate still additional structure-function relationships. These two particular relationships represent the converse of each other and are demonstrated in normal BALB/c mice treated with IL-1, granulocyte colony-stimulating factor (G-CSF) or IFN-γ or IL-12 (Table 4).

Enhanced structure-normal function. Curry & Kaye (1992) have reported that injecting IL-1 into chronically infected B10.D2 mice increases liver granuloma number and cellularity. Nonetheless, this histological effect did not translate into enhanced antileishmanial activity, providing an additional example of conditions under which intact/enhanced-appearing granuloma structure does not correlate with function.

The developing granuloma in *L. donovani*-infected normal animals can also be repopulated with neutrophils and clearly expanded in size and cellularity by continuous treatment with human G-CSF (Figure 9) (Murray *et al*. 1995). Since neutrophils do not appear to play an antileishmanial role in the normal host already endowed with cell-mediated immune responses, it was not surprising that granuloma remodeling by G-CSF failed to induce antileishmanial activity. However, exogenous GM-CSF did enhance parasite killing; thus, pointing to the likely importance of stimulated influx of additional monocytes (rather than primarily granulocytes) and/or to GM-CSF's capacity to directly activate macrophages (Murray *et al*. 1995). Either explanation may be relevant to the different granuloma structure-function relationships induced by treatment with these two CSFs.

Normal structure-enhanced function. Treating normal two-week *L. donovani*-infected mice for seven days with either exogenous IL-12 (primarily to simulate IFN-γ secretion) or IFN-γ alone induces clear-cut leishmanicidal activity (Murray 1990; Murray & Hariprashad 1995). This treatment effect, however, is not associated with readily discernible histological changes in developing granulomas in the liver (Table 4). This discrepancy may relate to the already established induction of IL-12 and IFN-γ in infected normal mice and/or to the already initiated granulomatous response. Nevertheless, IFN-γ has been shown to influence T cell and monocyte recruitment in other models of inflammation (Murray 1999; Tannenbaum & Hamilton 2000), and as judged by the failure of *L. donovani*-parasitized Kupffer cells in IFN-γ KO mice to attract mononuclear cells (Figure 7), endogenous IFN-γ is probably active in this mechanism as well (Taylor & Murray 1997).

Evidence supporting exogenous IFN-γ's capacity to

promote granuloma assembly, however, could be demonstrated in partially reconstituted nude mice. While exogenous IFN-γ induces no effect in unreconstituted nude mice (its efficacy requires host T cells), in infected nude mice first given either CD4 or CD8 cells, IFN-γ treatment stimulated mononuclear cell influx and granuloma formation in the liver (Murray *et al*. 1995). In addition, the granuloma-inducing activity of IL-12 could also be uncovered in IL-12-treated IFN-γ KO mice (Taylor & Murray 1997), likely in part because endogenous IFN-γ's own inflammatory and recruitment effects were absent. In these latter mice, the partial action of exogenous IL-12 is by definition IFN-γ-independent.

Relationship 7: no structure-normal/enhanced function – the invisible granuloma in immune mice

Unlike the previous six structure-function relationships, this final relationship does not occur in the immune response to primary *L. donovani* infection, but instead is expressed in chronic visceral infection. As previously indicated, after near-resolution of initial infection in normal BALB/c mice by weeks 8–12, intracellular amastigotes persist in liver foci for >1 years in a quiescent state (Murray *et al*. 1992). This low-level infection does not reactivate, but residual amastigotes clearly retain the capacity to re-emerge and replicate if the normal host is rendered immunodeficient. For example, relapse can be provoked in the liver long after resolution of primary infection by repeated injections of mAb which deplete T cells (CD4 cells) or neutralize key activating cytokines (IFN-γ, IL-2, TNF) (Murray *et al*. 1996). These latter effects indicate the influence of intact T cell-dependent and cytokine-mediated responses in maintaining visceral *L. donovani* in a latent state and preventing recrudescence.

However, surprisingly, inspection of livers of BALB/c mice six to 12 months after primary infection shows no microscopically apparent histological reaction at all despite harboring viable intracellular amastigotes (Murray *et al*. 1992). Thus, this model of chronic infection points to effective antileishmanial function (maintenance of immunity, no relapse) in tissue devoid of any form of granuloma.

Additional support for the notion that granulomas may be entirely dispensible for antileishmanial host defence came from experiments in which the same previously infected BALB/c mice were used to test mechanisms of resistance to rechallenge. In this model, immune mice resist reinfection and show minimal increases in liver parasite burden during 14 days after rechallenge (Murray *et al*. 1992). This response, a solid example of the power of the memory T cell, is appropriately accompanied by the rapid appearance and markedly accelerated maturation of numerous granulomas at all infected foci in the liver. Treating immune mice before and after rechallenge with the global T cell-and Th1 cell cytokine-suppressant, cyclosporin A, completely abolished granuloma assembly; nonetheless, resistance to rechallenge was not significantly impaired (Murray *et al*. 1992). Thus, while this last set of observations may only be germane in a previously sensitized normal host with well-established acquired immunity, intact or enhanced antileishmanial activity in the absence of granulomas makes the case for this final, quite separate type of granuloma structure-function relationship.

Acknowledgements

This study was supported by NIH grant AI 16393.

References

Adams D.H. & Lloyd A.R. (1997) Chemokines. leucocyte recruitment and activation cytokines. *Lancet* **349**, 490–495.

Adams D.O. (1976) The granulomatous inflammatory response. *Am. J. Pathol.* **84**, 164–191.

Anonymous. (1991) Granulomas and cytokines. *Lancet* **337**, 1067–1068.

Babaloo Z., Kaye P.M. & Eslami M.B. (2001) IL-13 in Iranian patients with visceral leishmaniaisis: relationship to other Th2 and Th1 cytokines. *Trans. Roy. Soc. Trop. Med. Hyg.* **95**, 1–4.

Baker R., Chiodini P. & Kaye P.M. (1999) Leishmaniasis, In: *The Granulomatous Disorders* (eds D. Geraint James & A. Zumla), pp. 212–234. Cambridge University Press, Cambridge, UK.

Bluestone J.A. (1997) Is CTLA-4 a master switch for peripheral T cell tolerance? *J. Immunol.* **158**, 1989–1993.

Cervia J., Rosen H. & Murray H.W. (1993) Effector role of blood monocytes in experimental visceral leishmaniasis. *Infect. Immun.* **61**, 1330–1333.

Correa E.B., Cunha J.M.T., Bunn-Moreno M.M. & Madeira E.D. (1992) Cyclophosphamide affects the dynamics of granuloma formation in experimental visceral leishmaniasis. *Parasitol. Res.* **78**, 154–160.

Cotterell S.E., Engwerda C. & Kaye P.M. (1999) *Leishmania donovani* infection initiates T cell-independent chemokine responses which are subsequently amplified in a T cell-dependent manner. *Eur. J. Immunol.* **29**, 203–214.

Curry A. & Kaye P.M. (1992) Recombinant interleukin-1α augments granuloma formation but not parasite clearance in mice infected with *Leishmania donovani*. *Infect. Immun.* **60**, 4422–4424.

Daneshbod K. (1972) Visceral leishmanaisis (kala-azar) in Iran: a pathologic and electron microscopic study. *Am. J. Clin. Pathol.* **157**, 156–166.

Di Perri G., Bonora S., Allegranzi B. & Concia E. (1999) Granulomatous inflammation and transmission of infectious disease. *Immunol. Today* **20**, 337–338.

D'Souza C.D., Cooper A.M., Frank A.A. *et al.* (2000) A novel

nonclassic B2-microglobulin-restricted mechanism influencing early lymphocyte accumulation and subsequent resistance to tuberculosis in the lung. *Am. J. Respir. Cell. Mol. Biol.* **23**, 188–193.

EHLERS S., KUTSCH S., BENINI J. *ET AL.* (1999) NOS2-derived nitric oxide regulates the size, quantity and quality of granuloma formation in *Mycobacterium avium*-infected mice without affecting bacterial loads. *Immunology* **98**, 313–323.

EL HAG I.A., HASHIM F.A., EL TOUM A., HOMEIDA M., L KALIFA M. & EL HASSAN A.M. (1994) Liver morphology and function in visceral leishmaniasis. *J. Clin. Pathol.* **47**, 547–551.

ENGWERDA C.R., SMELT S.C. & KAYE P.M. (1996) An *in vivo* analysis of cytokine production during *Leishmania donovani* infection in *scid* mice. *Exp. Parasitol.* **84**, 195–202.

ENGWERDA C.R., MURPHY M.L., COTTERELL S.E., SMELT S.C. & KAYE P.M. (1998) Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of *Leishmania donovani*. *Eur. J. Immunol.* **28**, 669–680.

FALLON P.G. (2000) Immunopathology of schistosomiasis. a cautionary tale of mice and men. *Immunol. Today* **21**, 29–35.

GAZZINELLI R.T., WYSOCKA M., HIENY S. *ET AL.* (1996) In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-γ and TNF-α. *J. Immunol.* **157**, 798–805.

GOMES N.A., GATTASS C.R., BARRETO-D., E-SOUZA V., WILSON M.E. & DOSREIS G.A. (2000) TGF- mediates CTLA-4 suppression of cellular immunity in murine kalaazar. *J. Immunol.* **164**, 2001–2008.

GROUX H., CITTREZ F., ROULEAU M. *ET AL.* (1999) A transgenic model to analyze the immunoregulatory role of IL-10 secreted by antigen-presenting cells. *J. Immunol.* **162**, 1723–1729.

GUTIERREZ Y., MAKSEM J.A. & REINER N.E. (1984) Pathologic changes in murine leishmaniasis (*Leishmania donovani*) with special reference to the dynamics of granuloma formation in the liver. *Am. J. Pathol.* **114**, 222–230.

HERNANDEZ-PANDO R., BORNSTEIN Q.L., AGUILAR LEON D., OROZCO E.H., MADRIGAL V.K. & MATRINEZ CORDERO E. (2000) Inflammatory cytokine production by immunological and foreign body multinucleated giant cells. *Immunology* **100**, 352–358.

HESSE M., CHEEVER A.W., JANKOVIC D. & WYNN T.A. (2000) NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. *Am. J. Pathol.* **157**, 945–955.

LAURENTI D.M., SCOTTO M.N., CORBETT C.E.P. *ET AL.* (1990) Experimental visceral leishmaniasis: sequential events of granuloma formation at subcutaneous inoculation site. *Int. J. Exp. Pathol.* **71**, 791–797.

LUSTER A.D. (1998) Chemokines – chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* **338**, 436–445.

MARINO M.W., DUNN A., GRAIL M. *ET AL.* (1997) Characterization of tumor necrosis factor- deficient mice. *Proc. Natl. Acad. Sci. USA* **94**, 8093–8098.

MARQUEZ G. & MARTINEZ A.C. (2001) Chemokine: the times are a-changin′. *J. Clin. Invest.* **107**, 791–792.

MCELRATH J., MURRAY H.W. & COHN Z.A. (1988) Dynamics of granuloma formation in experimental visceral leishmaniasis. *J. Exp. Med.* **167**, 1927–1937.

MIELKE M.E., PETERS C. & HAHN H. (1997) Cytokines in the induction and expression of T-cell-mediate granuloma formation and protection in the murine model of listeriosis. *Immunol. Rev.* **158**, 79–93.

MELBY P.C., YANG Y.-Z., CHENG J. & ZHAO W. (1998) Regional differences in the cellular immune response to experimental cutaneous or visceral infection with Leishmania donovani. *Infect. Immun.* **66**, 18–27.

MIRALLES G.D., STOECKLE M.Y., MCDERMOTT D.F., FINKELMAN F.D. & MURRAY H.W. (1994) Induction of Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. *Infect. Immun.* **62**, 1085–1063.

MORENO A., MARAZUELA M., YEBRA M. *ET AL.* (1988) Hepatic fibrin-ring granulomas in visceral leishmaniasis. *Gastroenterology* **95**, 1123–1126.

MURPHY M.L., COTTERELL S., GORAK M., ENGWERDA C.R. & KAYE P.M. (1998) Blockade of CTLA-4 enhances host resistance to the intracellular pathogen, *Leishmania donovani*. *J. Immunol.* **161**, 4153–4160.

MURRAY H.W., STERN J., WELTE K., RUBIN B.Y., CARRIERO S.M. & NATHAN C.F. (1987) Experimental visceral leishmaniasis. production of interleukin 2 and gamma interferon, tissue immune reaction, and response to treatment with interleukin 2 and gamma interferon. *J. Immunol.* **38**, 2290–2297.

MURRAY H.W. (1990) Effect of continuous administration of interferon-gamma in experimental visceral leishmaniasis. *J. Infect. Dis.* **161**, 992–994.

MURRAY H.W., SQUIRES K.E., MIRALLES G.D. *ET AL.* (1992) Acquired resistance and granuloma formation in experimental visceral leishmaniasis: differential T cell and lymphokine roles in initial vs. established immunity. *J. Immunol.* **148**, 1858–1865.

MURRAY H.W., MIRALLES G.D., STOECKLE M.Y. & MCDERMOTT D.F. (1993) Role and effect of interleukin-2 in experimental visceral leishmaniasis. *J. Immunol.* **151**, 929–934.

MURRAY H.W. & HARIPRASHAD J. (1995) Interleukin 12 is effective treatment for an established systemic intracellular infection. experimental visceral leishmaniasis. *J. Exp. Med.* **181**, 387–391.

MURRAY H.W., HARIPRASHAD J., AGUERO B., ARAKAWA T. & YEGANEGI H. (1995) Intracellular antimicrobial response of the T cell-deficient host to cytokine therapy. effect of interferon-gamma in experimental visceral leishmaniasis in nude mice. *J. Infect. Dis.* **171**, 1309–3016.

MURRAY H.W., CERVIA J., HARIPRASHAD J., TAYLOR A.P., STOECKLE M.Y. & HOCKMAN H. (1995) Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. *J. Clin. Invest.* **95**, 1183–1189.

MURRAY H.W., HARIPRASHAD J. & FICHTL R. (1996) Models of relapse of experimental visceral leishmaniasis. *J. Infect. Dis.* **173**, 1041–1043.

MURRAY H.W. (1997) Endogenous IL-12 regulates acquired resistance in experimental visceral leishmaniasis. *J. Infect. Dis.* **175**, 1477–1479.

MURRAY H.W., HARIPRASHAD J. & COFFMAN R.L. (1997) Behavior of visceral *Leishmania donovani* in an experimentally-induced Th2 cell-associated response model. *J. Exp. Med.* **185**, 867–874.

MURRAY H.W. (1999) Granulomatous inflammation: host antimicrobial defense in the tissues in visceral leishmaniasis. In: *Inflammation: Basic Principles and Clinical Correlates* (eds J. GALLIN, R. SYNDERMAN, D. FEARON, B. HAYNES, C. NATHAN) 3rd edn, pp. 977–994. Lippincott-Raven, Philadelphia, PA.

MURRAY H.W. & NATHAN C.F. (1999) Macrophage microbicidal

mechanisms in vivo. reactive nitrogen vs. oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. *J. Exp. Med.* **189**, 741–746.

MURRAY H.W. (2000a) Treatment of visceral leishmaniasis (kala-azar). a decade of progress and future approaches. *Int. J. Infect. Dis.* **4**, 158–177.

MURRAY H.W. (2000b) Mononuclear cell recruitment, granuloma assembly, and response to treatment in experimental visceral leishmaniasis. intracellular adhesion molecule 1-dependent and -independent regulation. *Infect. Immun.* **68**, 6294–6299.

MURRAY H.W. & DELPH-ETIENNE S. (2000) Role of endogenous gamma interferon and macrophage microbicidal mechanisms in host response to chemotherapy in experimental visceral leishmaniasis. *Infect. Immun.* **68**, 288–293.

MURRAY H.W., JUNGBLUTH A., RITTER E., MONTELIBANO C. & MARINO M.W. (2000a) Visceral leishmaniasis in mice devoid of tumor necrosis factor and response to treatment *Infect. Immun.* **68**, 6289–6293.

MURRAY H.W., MONTELIBANO C., PETERSON R. & SYPEK J.P. (2000b) Interleukin 12 regulates the response to chemotherapy in experimental visceral leishmaniasis. *J. Infect. Dis.* **182**, 1497–1502.

MURRAY H.W. (2001) Clinical and experimental advances in treatment in visceral. leishmaniasis. *Antimicrob. Agents Chemother.* (in press).

PAMPIGLIONE S., LA PLACA M. & SCHLICK G. (1974a) Studies on Mediterranean leishmanaisis. II. An outbreak of visceral leishmaniasis in northern Italy. *Trans. Roy. Soc. Trop. Med. Hyg.* **68**, 349–359.

PAMPIGLIONE S., MANSON-BAHR P.E.C., GIUNTI F., GIUNTI G., PARENTI A. & TROTTI C.G. (1974b) Studies on Mediterranean leishmaniasis. II. Asymptomatic cases of visceral leishmaniasis. *Trans. Roy. Soc. Trop. Med. Hyg.* **68**, 447–453.

REINER S.L. & LOCKSLEY R.M. (1995) The regulation of immunity to *Leishmania major*. *Annu. Rev. Immunol.* **13**, 151–177.

ROACH T.I., KIDERLEN A.F. & BLACKWELL J.M. (1991) Role of inorganic nitrogen oxides and tumor necrosis factor-alpha in killing *Leishmania donovani* amastigotes in gamma-interferon lipopolysaccharide-activated macrophages from *Lsh*^{s} and *Lsh*^{r} congenic mouse strains *Infect. Immun.* **59**, 3935–3944.

SATOSKAR A.R., RODIG S., TELFORD S.R. *ET AL.* (2000) IL-12 gene-deficient C57BL/6 mice are susceptible to *Leishmania donovani* but have diminished hepatic pathology. *Eur J. Immunol.* **30**, 834–839.

SAUNDERS B.M. & COOPER A.M. (2000) Restraining mycobacteria. role of granulomas in mycobacterial infections. *Immunol. Cell Biol.* **78**, 334–341.

SHEFFIELD E.A. (1990) The granulomatous inflammatory response. *J. Pathol.* **160**, 1–2.

SPELLBERG B. & EDWARDS J.E.(2001) Type 1/type 2 immunity in infectious diseases. *Clin. Infect. Dis.***32**, 76–102.

SQUIRES K.E., SCHREIBER R.D., MCELRATH M.J. & MURRAY H.W. (1989) Experimental visceral leishmaniasis. role of endogenous interferon-gamma in host defense and tissue granulomatous response. *J. Immunol.* **143**, 4244–4249.

STERN J.J., OCA M.J., RUBIN B.Y., ANDERSON S. & MURRAY H.W. (1988) Role of L3T4+ and Lyt-2+ cells in experimental visceral leishmaniasis. *J. Immunol.* **140**, 3971–3976.

TANNENBAUM C.S. & HAMILTON T.A. (2000) Immune-inflammatory mechanisms in IFN- γ-mediated anti-tumor activity. *Cancer Biol.* **10**, 113–123.

TAPIA F.J., CACERES-DITTMAR G., SANCHEZ M.A., FERNANDEZ C.T., RONDON A.J. & CONVIT J. (1994) Adhesion molecules in lesions of American cutaneous leishmaniasis. *Exp. Dermatol.* **3**, 17–22.

TAYLOR A. & MURRAY H.W. (1997) Intracellular antimicrobial activity in the absence of interferon-γ. effect of interleukin 12 in experimental visceral leishmanaisis in interferon-γ gene-disrupted mice. *J. Exp. Med.* **185**, 1231–1239.

TUMANG M., KEOGH C., MOLDAWER L.L., TEITELBAUM R.F., HARIPRASHAD J. & MURRAY H.W. (1994) The role and effect of tumor necrosis factor-alpha in experimental visceral leishmaniasis. *J. Immunol.* **153**, 768–775.

VERESS B., OMER A., SATIR A.A. & EL HASSAN A.M. (1977) Morphology of spleen and lymph nodes in fatal visceral leishmaniais. *Immunology* **33**, 605–610.

WARREN K.S. (1976) A functional classifiction of granulomatous inflammation. *Ann. N.Y. Acad. Sci.* **278**, 7–12.

WILLIAMS G.T. & WILLIAMS W.J. (1983) Granulomatous inflammation – a review. *J. Clin. Pathol.* **36**, 723–733.

WILSON M.E., DE INNES D.J.Q., SOUSA A. & PEARSON R.D. (1987) Early histopathology of experimental infection with *Leishmania donovani* in hamsters. *J. Parasitol.* **73**, 55–63.

WILSON M.E., SANDOR M., BLUM A.M. *ET AL.* (1996) Local suppression of IFN-γ in hepatic granulomas correlates with tissue-specific replication of *Leishmania chagasi*. *J. Immunol.* **156**, 2231–2239.

WILSON M.E., YOUNG B.M., DAVIDSON B.L., MENTE K.A. & MCGOWAN S.E. (1998) The importance of TGF- in murine visceral leishmaniasis. *J. Immunol.* **161**, 6148–6155.